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Next-generation sequencing of hereditary hemochromatosis-related genes: Novel likely pathogenic variants found in the Portuguese population

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ABSTRACT

Hereditary hemochromatosis (HH) is an autosomal recessive disorder characterized by excessive iron absorption resulting in pathologically increased body iron stores. It is typically associated with common *HFE* gene mutation (p.Cys282Tyr and p.His63Asp). However, in Southern European populations up to one third of HH patients do not carry the risk genotypes.

This study aimed to explore the use of next-generation sequencing (NGS) technology to analyse a panel of iron metabolism-related genes (*HFE*, *TFR2*, *HJV*, *HAMP*, *SLC40A1*, and *FTL*) in 87 non-classic HH Portuguese patients.

A total of 1241 genetic alterations were detected corresponding to 53 different variants, 13 of which were not described in the available public databases. Among them, five were predicted to be potentially pathogenic: three novel mutations in *TFR2* [two missense (p.Leu750Pro and p.Ala777Val) and one intronic splicing mutation (c.967-1G>C)], one missense mutation in *HFE* (p.Tyr230Cys), and one mutation in the 5′-UTR of *HAMP* gene (c.-25G>A).

The results reported here illustrate the usefulness of NGS for targeted iron metabolism-related gene panels, as a likely cost-effective approach for molecular genetics diagnosis of non-classic HH patients. Simultaneously, it has contributed to the knowledge of the pathophysiology of those rare iron metabolism-related disorders.

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1. Introduction

Hereditary hemochromatosis (HH; OMIM #235200) is an adult-onset autosomal recessive disorder, common among Caucasians of Northern European ancestry, which leads to progressive accumulation of iron in parenchymal cells of multiple organs that can lead, ultimately, to multi-systemic damage, if left untreated. HH is prevalently due to a founder missense mutation, p.Cys282Tyr (c.845G>A) in the *HFE* gene (6p21.3) [1]. This mutation, in the homozygous state, remains the most frequent HH patients' genotype. However, particularly in Southern European populations, around one third of the patients with primary iron overload do not present that genotype or the compound heterozygosity for the p.Cys282Tyr and the p.His63Asp mutations [2]. In fact, in addition to *HFE*, other iron metabolism-related genes may be involved in HH development. The systemic regulation of iron homeostasis is fundamentally achieved through the hepcidin/ferroportin axis. Under homeostasis, hepcidin, a liver-secreted hormone, regulates the efflux of iron from cells through its interaction with ferroportin, the only known cellular iron exporter. While hepcidin control is relatively well known, the regulation of hepcidin expression is a multifaceted mechanism [3]. Consequently, there are different forms of HH resulting from mutations in genes either involved in the hepcidin/ferroportin axis or in the regulation of hepcidin expression: the HH type 2a and 2b (OMIM#602390 and #61333), also known as Juvenile Hemochromatosis (JH), are due to mutation in *HJV* and *HAMP* genes, respectively; HH type 3 (OMIM#604250), which is a disease mainly of adult onset but where some juvenile forms can also be described, is characterized by genetic mutations in the *TFR2* gene, and HH type 4 (OMIM#606069) is due to mutations in the iron exporter ferroportin (*SLC40A1* gene).

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Furthermore, in addition to the common *HFE* mutations, several rare variants of this gene have also been associated with HH [4–9].

With the advent of massive parallel DNA sequencing, such as the next-generation sequencing (NGS), a number of challenges in the molecular genetics diagnosis of non-classic HH can be addressed. This study aimed to explore the use of a fully customized amplicon-based assay for targeted resequencing by NGS a panel of six HH-related genes in patients presenting clinical and biochemical features compatible with HH but in which the classic HH-associated *HFE* genotypes were not found. This way, several rare and novel variants were detected. Subsequently, *in silico* as well as genotype/phenotype correlation studies have facilitated the interpretation of the likely etiologic significance of some of the previously undescribed variants. Therefore, it was possible to propose the novel variants play a pathogenic role in different types of HH.

The results gathered in this study allowed to conclude that the merging of TruSeq Custom Amplicon (TSCA) methodology and NGS technology can be applied to facilitate the detection of rare variants associated with non-classic HH and, ultimately, to contribute to the understanding of the pathophysiology of those clinical conditions.

2. Materials and methods

2.1. Sample characterization

Patients enrolled in this study were selected by clinicians due to their persistent increased iron status biomarkers, including serum ferritin >300 μg/L and transferrin saturation > 60%, absence of evident environmental risk factors for secondary iron overload (such as, alcohol and hepatitis), and for having a negative first level HH genetic test (which means, absence of homozygosity for the *HFE* p.Cys282Tyr mutation or of compound heterozygosity for p.Cys282Tyr and p.His63Asp).

Following appropriate informed consent, the study was performed in 87 Portuguese patients (69 male and 18 female), presenting a mean age of 51 years and a mean serum levels of iron-related parameters of: iron 185 μg/dL, ferritin 940 μg/L, and transferrin saturation 76%.

Peripheral blood samples were collected in EDTA and used for DNA extraction in a MagNA Pure nucleic acid extractor (Roche). DNA fluorometric quantitation assays were performed in a Qubit™ equipment (Invitrogen) as recommended by the manufacturer. DNA sample concentrations were normalised to 25 ng/μL using purified bidistilled water.

An extra set of five DNA samples previously analysed by Sanger sequencing and known to present a total 43 genetic alterations corresponding to 14 different genetic variants in *HFE*, *TFR2*, *HAMP*, and *SLC40A1* [8] were used as positive controls: *HFE* (rs1799945, rs2071303, rs200706856, rs1572982, and rs1800758); *TFR2* (rs41295912, rs148902192, rs80338885, and rs2075674); *HAMP* (c.-25G>A); *SLC40A1* (rs2304704, rs4287798, rs1156835, and rs13008848).

2.2. Panel design

A fully customizable, amplicon-based assay for targeted resequencing was designed using the Design Studio 1.7 software (Illumina). It is a user-friendly online tool that provides dynamic feedback to optimize design and region coverage. Automated data analysis using the Illumina Amplicon Viewer software allowed to easily reviewing the project data. The panel consisted of ninety-seven amplicons with an average of 250 bp in length, covering a cumulative target sequence of 12,115 bp including coding regions, exon/intron junctions, promoters and untranslated regions (UTRs) of *HFE*, *TFR2*, *HJV*, *HAMP*, *SLC40A1* and *FTL* genes. The location of the designed amplicons and their corresponding length are summarized in Supplementary Table I.

2.3. Next-generation sequencing

TSCA DNA libraries were prepared according to the manufacturer's instructions and sequenced on a MiSeq instrument (Illumina) using paired-end reads. Firstly, a training set of 5 samples (the positive controls) was used to optimize the entire process of target amplification and sequencing. In this way it was verified that no true variant was missed and that no other genetic alterations undetected by Sanger sequencing were observed. Then, the 87 uncharacterized DNA samples were analysed. Sequence variants were identified using the built-in data analysis workflow of the *MiSeqReporter* software (Illumina). Variants were called against the human genome reference hg19 for genomic coordinates contained in the manifest file. Each variant was analysed in respect to the total coverage (TC), quality (QUAL), variant frequency (VF) and genotype (GT). A TC of 20 was used as a threshold for the acceptance of variants. Also an analysis of individual reads was made using the *Integrative Genomics Viewer* software (http://www.broadinstitute.org/igv/). The identified variants classified as likely pathogenic were confirmed by Sanger sequencing using the BigDye Terminator v1.1 Cycle sequencing kit (Applied Biosystems) and a 3130XL Genetic Analyser (Applied Biosystems).

2.4. Bioinformatics studies

Genetic variants were considered novel when not annotated in any of the following public databases: *ENSEMBL* (http: www.ensembl.org/index.html), *dbSNPs* (http://www.ncbi.nlm.nih.gov/), *GeneCards* (http://www.genecards.org/), and *1000 Genomes* (http://www.1000genomes.org).

Novel variants were named according to the *Human Genome Variation Society* (HGVS) recommendations (http://www.hgvs.org/mutnomen/recs.html). Bioinformatics tools were used to predict their deleterious effect. In the case of missense mutations the putative effect on protein structure and function, as well as the study of multiple sequence alignment were analysed using the *Polyphen-2* software (http://genetics.bwh.havard.edu/pph2) and the *HumDiv* and *HumVar* models. *Polyphen-2* prediction is based on a number of features comprising the sequence, phylogenetic and structural information characterizing the substitution. Also, the software *Sorting Intolerant From Tolerant* (SIFT) which predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids, was applied [10]. In what concerns splicing variants their putative effect on the corresponding pre-mRNA splicing was studied using the *Human Splicing Finder, vs 3.0* software (http://www.umd.be/HSF/). The *MaxEntScan* matrice based on the *Maximum Entropy Principle* was also used. It is based on the approach for modelling the sequences of short sequence motifs such as those involved in RNA splicing which simultaneously accounts for non-adjacent as well as adjacent dependencies between positions. This method is based on the most previous probabilistic models of sequence motifs such as weight matrix models and inhomogeneous Markov models [11].

3. Results

3.1. Validation of the amplicon panel for next-generation sequencing studies

A gene panel was designed in order to generate 97 amplicons, including exons, intron/exon junctions, promoters and UTRs of of six genes that we routinely screen by Sanger sequencing: *HFE*, *TFR2*, *HJV*, *HAMP*, *SLC40A1* and *FTL* (Supplementary Table I). The amplified targets of approximately 250 bp were sequenced on the MiSeq equipment (Illumina) and the resultant paired-end reads were analysed through the built-in analysis pipeline. The sequences were aligned against human genome reference hg19 using alignment and variant caller algorithms in the *MiSeqReporter* software. Firstly, five known DNA positive

controls were used to evaluate and validate the TSCA kit and the performance of the NGS workflow. These positive DNA controls contain a total of 43 alterations corresponding to 14 different known genetic variants in *HFE*, *TFR2*, *HAMP* and *SLC40A1* genes. All genetic variants were correctly identified with 100% of concordance with Sanger sequencing results. However, 1 out of the 97 amplicons (≈1% in the panel) failed to produce any reads (the amplicon no.33, corresponding to exon 17 of *TFR2*, presented in the Supplementary Table I). Thus, this exon was later analysed in all samples by Sanger sequencing.

3.2. Patients' genetic variants identification and validation

Subsequently to the validation phase, the 87 DNAs from the iron overload patients enrolled in this study matching the previously established inclusion criteria were sequenced at high coverage on the MiSeq equipment maintaining the same experimental conditions as used for controls. Through this approach a cumulative target sequence of 12,115 bp were obtained. A total of 1241 molecular alterations were detected, corresponding to 53 different genetic variants: 14 missense, 8 synonymous, 5 located at splicing regions, 1 premature translation initiation codon, 1 located within an Iron-Responsive Element (IRE), and 24 common SNPs known to integrate haplotypes in some of the mentioned genes (Table I and Table II). Thirteen out of the mentioned 53 different identified variants were absent from the public databases (Table I). One of them, the *HAMP* (c.-25G>A), although not annotated in databases had been already found in the Portuguese population [8,12,13], and was considered a private variant. Among these 13 novel/private variants, five were predicted to be pathogenic by *in silico* studies and by genotype/phenotype correlation: three are missense mutations in *HFE* (p.Tyr 230Cys), and *TFR2* (p.Leu750Pro and p.Ala777Val); one is a splicing mutation in *TFR2* gene (c.967-1G>C); and one is located at the 5′-UTR of the *HAMP* gene (c.-25G>A). Furthermore, another novel variant was found in a conserved regions of the IRE (c.-173C>G of *FTL* gene (Table I). All these variants were validated by Sanger sequencing in separate PCR products (Supplementary Fig. 1).

3.3. In silico studies of pathogenicity of the novel variants

The putative pathogenicity of the three novel missense mutations was analysed using the bioinformatics tools, *PolyPhen-2* and the *HumDiv* and *HumVar* models as well as SIFT. Concerning the mutation c.689A>G in exon 4 of *HFE* gene, p.Tyr230Cys in HFE protein, it was found in heterozygosity in a 51-year-old man presenting serum iron = 278 μg/dL, ferritin = 651 ng/mL and transferrin saturation = 89%. This mutation was predicted by *PolyPhen-2* to cause a possibly damaging effect at the protein level because it presented a score of 0.857 [0.941 by *HumDiv* (sensitivity 0.80; specificity 0.94) and 0.745 by *HumVar* (sensitivity 0.77; specificity 0.86)]. Concerning SIFT prediction it was classified as deleterious at protein level with the score of 0. In addition, HFE amino acid multiple sequence alignment around Tyr230, performed in *PolyPhen-2*, has shown that this amino acid residue is conserved in 9 out 10 of the analysed mammalian species.

The missense mutation c.2249T>C located at exon 18 of *TFR2* gene (p.Leu750Pro) was found in homozygosity in a 56-year-old man, presenting the following iron status: serum iron = 260 μg/dL, serum ferritin = 2961 ng/mL and transferrin saturation of 84%. This variant was predicted by *PolyPhen-2* as probably damaging the protein because presented a score of 0.966 [0.963 by *HumDiv* (sensitivity 0.78; specificity 0.95) and 0.837 by *HumVar* (sensitivity 0.73; specificity 0.88)]. The SIFT prediction was deleterious (score 0). The multiple sequence alignment by *PolyPhen-2* revealed that the p.Leu750 is invariable between species which is also in accordance with the hypothesis that this mutation has a pathogenic effect on protein structure/function.

Concerning the other *TFR2* variant also located within exon 18, *TFR2*: c.2330C>T, p.Ala777Val, it was detected in two patients. It was found in double heterozygosity with the p.His63Asp in a 36-year-old man presenting a serum iron = 236 μg/dL and transferrin saturation of 86%, and it was found in heterozygosity in a 65-year-old woman presenting serum iron = 197 μg/dL and transferrin saturation = 73%. This mutation was predicted by *PolyPhen-2* to generate a probably damaging effect at protein level with the highest score of 1, including the scores of 1.000 by *HumDiv* and a score of 0.999 by *HumVar*. SIFT prediction was deleterious with a score of 0. Also, TfR2 amino acid multiple sequence alignment around Ala777, performed in *PolyPhen-2*, has shown this amino acid is invariable between the studied 10 mammalian species.

In relation to the novel *TFR2* splicing mutation, c.967-1G>C, it was found in homozygosity in a 69-year-old man presenting: serum iron = 198 μg/dL, ferritin = 1930 ng/mL, transferrin saturation = 100%. This variant is located in the last nucleotide of intron 7 of *TFR2* gene affecting this acceptor splice site. The bioinformatics tool *Human Splicing Finder* provided by the HSF matrices a score of 98.12 for the native acceptor sequence and a score of 69.18 for the mutant one. Thus, it is observed a reduction of 30% of variation in the sequence recognition scores, suggesting the wild type site broken and a deleterious effect on splicing. As well, the *MaxEntScan* matrices gave for the wild type sequence a score of 10.41 and for the mutant one a score of 2.34 with a −77.52% of variation.

Table I
Novel or private variants in iron metabolism-related genes found by NGS in this study.

Genomic location (hg19)	Gene	NGS allelic depth	Zygosity	NGS Qual	Genomic context	Nucleotide change	Amino acid change	Variant classification by *in silico* studies		
								Polyphen-2	*SIFT*	*HSF*
Chr6:26092985A>G	*HFE*	430:455	het	9069.08	Exon 4	c.689A>G	p.Tyr230Cys	Possibly damaging	Deleterious	
Chr6:26092801T>C	*HFE*	57:66	het	1325.07	Intron 3	c.617-112T>C				Likely benign
Chr7:100229569C>G	*TFR2*	0:453	hom	10655.17	Intron 7	c.967-1G>C				Probably damaging
Chr7:100218637A>G	*TFR2*	4:215	hom	4960.62	Exon 18	c.2249T>C	p.Leu750Pro	Probably damaging	Deleterious	
Chr7:100218556G>A	*TFR2*	141:116 141:146	het het	2236.37 4408.53	Exon 18	c.2330C>T	p.Ala777Val	Probably damaging	Deleterious	
Chr19:35773456G>A	*HAMP*	0:394 2:476	hom hom	15985.68 19146.68	5′UTR	c.−25G>A	Alters ORF	Pathogenic	Deleterious	
Chr19:35773318G>A	*HAMP*	62:49	het	874.57	5′UTR	c.-92G>A				Likely benign
Chr1:145414708G>A	*HJV*	240:218	het	4285.30	5′UTR	c.-74C>T				Likely benign
Chr1:145415253G>A	*HJV*	47:44	het	814.49	Intron 3	c.1002-26C>T				Likely benign
Chr1:145415769A>G	*HJV*	59:62	het	1198.75	Exon 4	c.932T>C	p.Asn129=	Likely benign		
Chr2:190436342A>G	*SLC40A1*	242:180	het	3470.70	Intron 5	c.514+99T>C				Likely benign
Chr19:49468592C>G	*FTL*	39:36	het	653.68	IRE_ 5′UTR	c.-173C>G				Uncertain significance
Chr19:49469233T>G	*FTL*	138:164	het	3347.00	Intron 2	c.249+60T>G				Likely benign

Table II
Genetic variants (already reported) in iron metabolism-related genes found by NGS in this study.

Gene	Variant ID	Genomic location	Nucleotide change	Genomic context	Clinical significance (Ensembl or ClinVar)
HFE					
	rs149342416	6:g.26087458G>C	c.18G>C	p.Arg6Ser	Benign; uncertain significance
	rs62625319	6:g.26087551G>A	c.76+35G>A	Intron variant	Likely benign
	rs1799945	6:g.26090951C>G	c.187C>G	p.His63Asp	Pathogenic; risk factor
	rs1800730	6:g.26090957A>T	c.193A>T	p.Ser65Cys	Pathogenic
	rs2071303	6:g.26091108T>C	c.340+4T>C	Splice region variant	Uncertain significance
	rs200706856	6:g.26091358G>A	c.385G>A	p.Asp129Asn	Probably Pathogenic [8]
	without ID	6:g.26092757A>T	c.689A>T	p.Tyr230Phe	Likely benign [8]
	rs1800562	6:g.26092913G>A	c.845G>A	p.Cys282Tyr	Pathogenic
	rs1800758	6:g.26093008G>A	c.892+48G>A	Intron variant	Likely benign
	rs2794717	6:g.26093069G>A	c.893-50G>A	Intron variant	Likely benign
	rs1800708	6:g.26093075T>C	c.893-44T>C	Intron variant	Likely benign
	rs201262562	6:g.26093246A>G	c.1006+14A>G	Intron variant	Likely benign
	rs1572982	6:g.26094139G>A	c.1007-47G>A	Intron variant	Likely benign
TFR2					
	rs376955913	7:g.100640856G>A	c.303C>T	p.Tyr101=	Synonymous/likely benign
	rs148902192	7:g.100640678A>T	c.473+8T>A	Splice region variant	Likely benign
	rs34242818	7:g.100633241G>C	c.714C>G	p.Ile238Met	Benign
	rs41303474	7:g.100631978G>A	c.967-33C>T	Intron variant	Likely benign
	rs41303501	7:g.100629279C>T	c.1364G>A	p.Arg455Gln	Uncertain significance; risk factor
	rs80338885	7:g.100628294C>T	c.1403G>A	p.Arg468His	Pathogenic
	rs139178017	7:g.100628224C>T	c.1473G>A	p.Glu491=	Synonymous/likely benign
	rs62625319	7:g.100627570G>A	c.1767+7C>T	Splice region variant	Likely benign
	rs2075674	7:g.100627408G>A	c.1851C>T	p.Arg617=	Synonymous/likely benign
	rs412295921	7:g.100627255C>G	c.1995+9G>C	Intron variant	Likely benign
	rs41295924	7:g.100627172C>T	c.1995+92G>A	Intron variant	Likely benign
	rs41295942	7:g.100621008C>T	c.2255G>A	p.Arg752His	Benign
HJV					
	rs56025621	1:g.146019740G>C	c.98-6C>G	Splice region variant	Uncertain significance
HAMP					
	rs142126068	19:g.35282425C>T	c.-153C>T	5′-UTR	Likely benign
	rs2293689	19:g.35284723C>T	c.91-66C>T	Intron variant	Likely benign
	rs104894696	19:g.35284999G>A	c.212G>A	p.Gly71Asp	Risk factor
SLC40A1					
	rs13008848	2:g.189580558C>G	c.-98G>C	5′-UTR	Likely benign
	rs11568351	2:g.189580468G>C	c.-8C>G	5′-UTR	Likely benign
	rs4287798	2:g.189580350T>G	c.43+68A>C	Intron variant	Likely benign
	rs1439816	2:g.189579904C>G	c.44-24G>C	Intron variant	Likely benign
	rs11568344	2:g.189572846G>A	c.387C>T	p.Leu129=	Synonymous/likely benign
	rs2304704	2:g.189565451A>G	c.663T>C	p.Val221=	Synonymous/likely benign
	rs185040528	2:g.189563529C>T	c.1402+55G>A	Intron variant	Likely benign
	rs73980217	2:g.189563522C>A	c.1402+62G>T	Intron variant	Likely benign
FTL					
	rs2230267	19:g.48965830T>C	c.163T>C	p.Leu55=	Synonymous/likely benign
	rs73046709	19:g.48966729C>T	c.521C>T	p.His174=	Synonymous/likely benign
	rs77793045	19:g.48966786G>T	c.*51G>T	3′-UTR	Likely benign

In this study, one mutation was found in the homozygous state in two siblings. The oldest sibling is a 49-year-old woman, presenting amenorrhea at age 32, arthralgia and hepatomegaly. Her brother is a 47-year-old man who exhibits hypogonadotrophic hypogonadism. Both patients present symptoms of iron overload since their youth and both started phlebotomies at 24 years of age. In genotypic terms, both patients are homozygotes for the *HAMP*: c.-25G>A variant located within the 5′-UTR of the *HAMP* gene (Table I). This variant is not annotated in the available public databases, however, it was already found in our population. This G to A substitution gives rise to a novel out-of-frame translation initiation codon (AUG), 25 nucleotides upstream to the native one, which greatly disturbs *HAMP* mRNA genetic information content [12,14].

It is noteworthy that although a NGS low coverage was obtained for the 5′-UTR region of *FTL*, one variant was detected in heterozygosity (allelic depth of 39:36, Table I) which was subsequently validated by Sanger sequencing, c.-173C>G (Supplementary Fig. 1). This was found in a 55-year-old man presenting serum ferritin = 1291 ng/mL, transferrin saturation = 84%. Cataracts have thus far not been reported.

None of the above described likely pathogenic gene variants presented in Table I was found in a group of 50 individuals (100 alleles) from the general Portuguese population so they should not be considered as common polymorphisms.

4. Discussion

In Northern European populations the high frequency of the HFE p.Cys282Tyr mutation makes the genetic diagnosis of HH relatively simple in the majority of the patients (a simple genetic test will confirm the diagnosis). However, genetic diagnosis of HH in Southern European populations may prove more challenging. The diversity and rarity of the mutations identified as causing iron overload have as consequence that a single molecular test in not sufficient to conclude the diagnosis. In fact, using current technologies, genetic diagnosis involves Sanger sequencing of the entire coding region of one or more of the known HH-related genes guided by phenotypic data. This is usually a costly and time-consuming procedure. The development of NGS, as a high throughput low-cost-per-base method, provided a much greater chance

of mutation identification in iron overloaded patients who present a negative *HFE*-first level genetic test.

In this study, we have successfully sequenced a panel of six iron metabolism-related genes, using 25 ng of gDNA per sequencing run and the amplicon panel designed by us provided the opportunity to analyse a wide range of samples per MiSeq run. In fact, we have processed 87 DNA samples plus the 5 positive DNA controls from the initial DNA sample to the analysed data in only a few days. Thus, TSCA provided an unprecedented level of sample multiplexing, including convenient online probe design and order, a stream line workflow and automatic data analysis. We can conclude that the implementation of NGS in research and subsequently as part of the routine clinical diagnosis may provide a comprehensive molecular genetics method with a clinically compatible throughput and turn-around time. However, establishing the clinical relevance of NGS-detected novel genetic variants in an autosomal recessive disorder with reduced penetrance and variable expressivity as HH, remains a difficult task, requiring further functional studies and international collaborative efforts. In addition, although this technology facilitates the detection of the genetic cause of iron overload in some patients with non-classic HH, there are still a few patients who have hereditary iron overload that are due to yet unidentified mutations in genes or combinations of genes that have yet to be discovered.

Concerning the novel variant p.Tyr230Cys in HFE, bearing in mind its location within the α3 domain of the protein and knowing this domain is crucial to HFE interaction with the chaperone β2-microglobulin [15], it is suggested that this change may generate an incorrect folding of the protein and a deficient presentation at the membrane surface, as was proven to occur in result of other mutations located within this domain. In fact, at least seven missense mutations causing disease were reported at the *HFE* exon 4 (which codes for the α3 domain of the protein) including the major p.Cys282Tyr mutation, making this exon a hot spot for mutations. In the case of this mutation, the tyrosine for cysteine substitution disrupts the formation of the disulfide bond that physiologically occurs between Cys225 and Cys282 residues and that is essential for HFE association with β2-microglobulin [1,16,17]. Therefore, the p.Cys282Tyr-mutated HFE protein is unable to bind to the chaperone β2-microglobulin and to be transported to cell surface where it would interact with TfR1 and TfR2 in order to regulate hepcidin expression. The same mechanism was proven to occur for some of the other missense mutations located at p.Cys282 neighbourhood, such as p.Gln238Pro, p.Glu277Lys, p.Val295Ala [18–20]. In our case, the p.Tyr230Cys-mutated HFE protein will probably allowed the creation of a new disulfide bridge between the Cys225 and the novel Cys230 which might disturb the correct HFE/β2-microglobulin binding and consequently the HFE correct folding and presentation at cell membrane.

TfR2-related HH (HH type 3) is characterized by increased intestinal iron absorption resulting in iron accumulation in the liver, heart, pancreas, and endocrine organs, as does HH type 1. The TfR2 protein is a transmembrane homodimer homolog of TfR1 and it is mainly expressed in the liver [21]. It is thought that TfR2 binds to HFE at cell surface and acts as a body iron sensor of diferric transferrin, resulting in the upregulation of hepcidin production through a not yet fully understood signalling pathway [22–25]. Concerning the novel variants in TfR2, in the case of p.Leu750Pro, proline is more hydrophilic than leucine and gives rise to a less flexible protein. Also, the p.Ala777Val seems to strongly perturb the protein structure and function because valine is an aliphatic and hydrophobic molecule that tends to favour the formation of helical structures. Moreover, both mutations are located within the dimerization domain of the TfR2 protein, near the carboxyl-terminal end, and were predicted by *PolyPhen-2* and SIFT as being deleterious. Several other missense variants located at this domain were classified as pathogenic, such as p.Gly792Arg, and p.Thr740Met, because the disruption of that domain is generally associated with protein severe loss of function [26,27]. In fact, this domain is crucial for the Fe_2-Tf-TfR2-HFE complex interactions. Nevertheless, another *TFR2* missense variant located in the same TfR2 domain (the c.2255G>A, p.Arg752His) is classified as a frequent polymorphism with a genetic modifier capacity [26,28,29]. In our study, the variant c.2255_ allele A was found with a frequency of 0.033 in the group of iron overloaded patients, whereas it was found with a frequency of 0.02 in 50 Portuguese individuals from the general population. However, this difference is not significant ($p = 0.892$; Qui-square test). A similar frequency has been also reported for the European general population, 0.021 (1000 Genomes project).

Also in the *TFR2* gene, we have found a novel splicing variant, the c.967-1G>C which is located in the last nucleotide of intron 7. This substitution significantly reduces the consensus value of the wild type splice site (−30% of variation by HSF and −78% by *MaxEnt*) meaning that this acceptor splice site is broken and cannot be recognised by the splicing machinery. Therefore, the presence of the variant may favour the exon 8 skipping and a consequent frameshift in the reading frame. As exon 8 is 140 nucleotides in long, its skipping originates a premature stop codon (PTC) at codon 304, as predicted by *Translated Tool of ExPASy* software (http://web.expasy.org/translate/), and may trigger the corresponding mRNA degradation by the nonsense mediated decay mechanism. Our patient presents this novel variant in homozygosity, *TFR2*: c.[967-1G>C)]; [967-1G>C)], which supports his HH type 3 phenotype. As far as we know, there are only four splicing mutations in *TFR2* gene classified as pathogenic and are associated with HH type 3: c.1606-8A>G, c.1538-2A>G, c.2137-1G>A, and c.614+4A>G [26–28,30,31].

Concerning the private *HAMP* c.-25G>A variant, it seems to have reached some prevalence in the Portuguese population. The first two cases of JH due to homozygosity for this mutation were reported by Matthes and co-workers [12] in two Portuguese siblings. Then, another two JH patients were reported, also presenting homozygosity for the same mutation, in two unrelated families of North [13] and Centre of Portugal [8]. In this study, we report another two Portuguese siblings, with JH phenotype and homozygosity for the same mutation. They descend from a first-cousin marriage in a village from Southern Portugal (Amareleja, Moura) and do not seem to be directly related with the first ones. This variant was not found in a group of 50 individuals (100 alleles) from the general Portuguese population, so it cannot be considered a common polymorphism.

Under physiological conditions, ferritin synthesis is finely regulated at the translational level by iron availability. It is achieved by high-affinity interaction between cytoplasmic mRNA binding proteins (the Iron-Regulatory Proteins, IRP) and a cis-acting non-coding mRNA stem-loop structure (the IRE). The 5′UTR of FTL mRNA presents an IRE where upon binding IRPs represses translation. We have found in the 5′-UTR of the *FTL* gene a variant (c.-173C>G) that is located at the 5′ stem of the IRE, position +27. As far as we know, there is no previously described single nucleotide substitution affecting this nucleotide. The only similar case was described in an Italian family with high serum ferritin levels and juvenile cataract. But, here the molecular lesion consisted in a six nucleotide deletion from positions +22 to +27 [32]. Our patient presented a severe iron overload phenotype which progressed to a fatal hepatocellular carcinoma. There was no report of cataracts in his clinical history. Thus, we cannot establish a clear genotype/phenotype correlation in this case. Also his relatives are not available to be recruited for a family study.

5. Conclusion

The recent development of NGS allows practical, manageable, and cost-effective analysis of the non-classic HH cases, proving to have significant utility when conventional testing has failed to identify the underlying molecular basis of the disease. As far as we know, only two studies have been published concerning the application of NGS to atypical iron disorders [33,34]. The identification and study of novel iron metabolism-related mutations are important steps forward to improve the knowledge of the HH genetic basis heterogeneity and of the pathophysiology of the different types of HH. Clinically, it is also important because

whilst the treatment of the more common forms of iron overload is similar, differential diagnosis remains important in atypical cases in which specific treatment and/or monitoring options are recommended.

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Authorship contributions

RF and BS performed the research laboratorial work, reviewed literature/databases and co-wrote the manuscript. CS performed library preparation and sequencing. PF designed the research study, reviewed the study results, performed genotype/phenotype correlations and wrote the manuscript. JG and PL collected data and performed the first level of molecular analysis; AQ, SF, JE, DM, and RF participated in clinical enrolling/work–up of HH patients. LV collaborated in the design of the sequencing panel and performed a critical revision of the manuscript. All authors revised and approved the manuscript final version.

Conflict of interests

The authors have no competing interests.

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